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Sequences of cDNAs and expression of genes encoding chitin synthase and chitinase in the midgut of *Spodoptera frugiperda*

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Abstract

The focus of this study was on the characterization and expression of genes encoding enzymes responsible for the synthesis and degradation of chitin, chitin synthase (*SfCHSB*) and chitinase (*SfCHI*), respectively, in the midgut of the fall armyworm, *Spodoptera frugiperda*. Sequences of cDNAs for *SfCHSB* and *SfCHI* were determined by amplification of overlapping PCR fragments and the expression patterns of these two genes were analyzed during insect development by RT-PCR. *SfCHSB* encodes a protein of 1523 amino acids containing several transmembrane segments, whereas *SfCHI* encodes a protein of 555 amino acids composed of a catalytic domain, a linker region and a chitin-binding domain. *SfCHSB* is expressed in the midgut during the feeding stages, whereas *SfCHI* is expressed during the wandering and pupal stages. Both genes are expressed along the whole midgut. Chitin staining revealed that this polysaccharide is present in the peritrophic membrane (PM) only when *SfCHSB* is expressed. There is little or no chitin in the midgut when *SfCHI* is expressed. These results support the hypothesis that SfCHSB is responsible for PM chitin synthesis during the larval feeding stages and SfCHI carries out PM chitin degradation during larval–pupal molting, suggesting mutually exclusive temporal patterns of expression of these genes.

Keywords: Fall armyworm; Peritrophic membrane; Chitin metabolism; Molting

1. Introduction

The peritrophic membrane (PM) is a hydrated anatomical structure that surrounds the food bolus in most insects. It is composed primarily of proteins and chitin (Peters, 1992), a non-ramified water-insoluble homopolymer of β-*N*-acetylglucosamine (GlcNAc). The PM is not only important for protection of insects against food abrasion and microorganisms, but it also plays a vital role in digestion (Terra, 2001; Terra and Ferreira, 2005). For example, a defective PM abolishes digestive enzyme recycling in midguts of larvae of the fall armyworm, *Spodoptera frugiperda* (Bolognesi et al., 2001), and increases mortality of *Trichoplusia ni* larvae (Wang and Granados, 2000).

Chitin is probably the most underexploited biomass resource available on earth and its metabolism in insect tissues is not well understood (Tharanathan and Kittur, 2003). In insects, cells present along the entire midgut or a specialized ring of cells associated with the cardia produce chitin. The main enzyme involved in chitin synthesis is chitin synthase (CHS) (EC 2.4.1.16, UDP-*N*-acetyl-D-glucosamine: chitin 4-β-*N*-acetylglucosaminyltransferase), which belongs to family 2 of the glycosyltransferases (Coutinho and Henrissat, 1999). This enzyme catalyzes the addition of *N*-acetylglucosamine to the growing chitin polymer using UDP-*N*-acetylglucosamine (UDP-GlcNAc) as substrate. Immunocytochemical analysis using wheat germ agglutinin or

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antibodies for either PM proteins or CHS revealed that the lepidopteran PM is primarily assembled in the anterior portion of the midgut where is expressed predominantly (Ryerse et al., 1992; Harper and Hopkins, 1997; Bolognesi et al. 2001; Zimoch and Merzendorfer, 2002).

The number of *CHS* genes in most insect species is likely to be two, based on an analysis of the *Drosophila* and *Anopheles* genome sequences. Based on tissue localization of *CHS* transcripts, it has been proposed that one of the CHS enzymes is responsible for synthesis of cuticular chitin (class A CHS), whereas the other produces the chitin associated with the PM (class B CHS) (Tellam et al., 2000; Zhu et al., 2002; Zimoch and Merzendorfer, 2002; Arakane et al., 2004; Hogenkamp et al., 2005; Zimoch et al., 2005). Southern blot analysis and BAC library screening with *CHS* gene probes have indicated that in the red flour beetle, *Tribolium castaneum*, there are also only two *CHS* genes, one encoding a class A (CHS-A) chitin synthase and the other encoding a class B (CHS-B) enzyme (Arakane et al., 2004). The presence of alternate exons is one of the unique features of genes encoding class A CHSs, a feature that has not been identified in any of the *CHS* genes encoding class B CHSs (Arakane et al., 2004; Hogenkamp et al., 2005).

Chitin is digested by chitinase (E.C. 3.2.1.14, poly [1,4-(*N*-acetyl-β-D-glucosaminide)] glycanohydrolase) to small soluble oligosaccharide products that are subsequently hydrolyzed to 2-acetamido-2-deoxyglucopyranoside by β-*N*-acetylglucosaminidases (Kramer and Muthukrishnan, 2005). In insects, chitinases are involved in cuticle turnover, nutrient digestion and PM degradation during molting. The products of chitin hydrolysis are recycled for the synthesis of new chitin (Lehane, 1997). Several chitinases from insects have been purified and their chemical, physical and kinetic properties were characterized (Kramer and Koga, 1986; Koga et al, 1992; Krishnan et al., 1994; Kramer and Muthukrishnan, 2005). The cloning of several chitinase cDNAs and genes has also been reported (reviewed in Kramer and Muthukrishnan, 2005).

Our understanding of the coordinated regulation of chitin synthesis and degradation in the insect midgut is limited at present. A detailed characterization of *CHS* and *CHI* genes and their expression in different developmental stages and specific tissues as well as studies of the functional significance of each gene are needed to clarify their precise roles during development. Moreover, chitin metabolism represents a potential target for selective biocidal agents because chitin is absent in plants and vertebrates (Kramer and Muthukrishnan, 1997, 2005; Kramer et al., 1997).

In this paper, we describe the cloning of *CHS* and *CHI* cDNAs from the midgut tissue of the fall armyworm *S. frugiperda*, as well as the expression patterns of these genes in the midgut during development. The roles of the CHS and CHI in midgut chitin metabolism, specifically PM formation and degradation are also discussed.

2. Material and methods

2.1. Cloning of S. frugiperda CHSB and CHI cDNAs

The chitin synthase cDNA cloned here was named *SfCHSB* because it is from midgut and shares the greatest sequence similarity with another lepidopteran class B CHS cDNA from *Manduca sexta* (see Section 3; Hogenkamp et al., 2005). To obtain the cDNA corresponding to *SfCHSB*, a cDNA library prepared from midguts of *S. frugiperda* feeding stage larvae in pBluescript (Marana et al., 2001) was used as template. For cloning of *SfCHSB* cDNA, we used a pair of degenerate primers corresponding to two conserved regions found in the catalytic domain of other insect CHSs. These degenerate primers were the same as those used by Arakane et al. (2004) for amplification of a region of a *CHSB* gene from *T. castaneum* encoding the segment in the catalytic domain between the conserved amino acid residues FEYAIGHW for the forward primer and another conserved sequence QYDQ-GEDRW for the reverse primer. To extend the sequences in either direction, additional degenerate primers corresponding to other conserved motifs of CHSs and gene-specific primers were used. The sequences of these primers are presented in Table 1. PCR reactions were carried out using the following conditions: denaturation at 94 °C for 1 min, annealing at 46–55 °C (depending on the primers used) for 1 min, and extension at 72 °C for 1–2 min for 25–30 cycles as indicated. The sequencing strategy used resulted in a series of overlapping PCR fragments that made it possible to assemble the DNA sequences coding for most of the midgut chitin synthase protein. To obtain the 5′-UTR sequence of *SfCHSB*, PCR was performed using DNA prepared using the midgut cDNA library as template and the gene-specific primer, 5′-TAAACGC TGTGACCAGAGAC-3′ (positions 635–654bp in the cDNA sequence, GenBank accession # AY525599), and the T3 primer, which corresponds to the T3 promoter contained in the pBluescript plasmid employed for construction of the cDNA library (Marana et al., 2001). To obtain the 3′-UTR sequence of cDNA, PCR was done with an oligo-(dT) adapter primer and the gene-specific forward primer, 5′-TCTCACACGCGTT GGAGAAG-3′ (positions 3380–3399 in the cDNA sequence).

For obtaining *SfCHI* cDNA, total RNA was isolated from midgut tissues of prepupae using the RNeasy Mini Kit (Qiagen) according to the manufacturer's

Table 1
Primers used for the amplification of cDNA corresponding to *SfCHSB* and *SfCHI*

cDNA	PCR fragment	Primer		
		Direction*	Type	Sequence
SfCHSB	1	F	Degenerate primer	ttygartaygcnathggncaytgg
SfCHSB	1	R	Degenerate primer	nckrtcytcnccytgrtcrtaytg
SfCHSB	2	F	Degenerate primer	tgygcnacnatgtggcayg
SfCHSB	2	R	Gene specific primer	tgcctcgtgggaagttaagg
SfCHSB	3	F	Gene specific primer	cgactgaacacatgattggc
SfCHSB	3	R	Degenerate primer	aanckrtgraanarcatngc
SfCHSB	4	F	Degenerate primer	cargaracnaarggntggga
SfCHSB	4	R	Gene specific primer	ccatgaagatgtgtacttcg
SfCHSB	5	F	5′ RACE Adapter	ggccacgcgtcgactagtac
SfCHSB	5	R	Gene specific primer	gtgtctactacgaaggccgt
SfCHSB	6	F	Gene specific primer	tctcacacgcgttggagaag
SfCHSB	6	R	3′ RACE Adapter	gaccacgcgtatcgatgtcga
SfCHI	1	F	Degenerate primer	gayytngaytgggartaycc
SfCHI	1	R	Degenerate primer	raartctccatrtcdatngc
SfCHI	2	F	5′ RACE Adapter	ggccacgcgtcgactagtac
SfCHI	2	R	Gene specific primer	tgaatgctctcctgagctcc
SfCHI	3	F	Gene specific primer	gctgtcccagtaacaagttg
SfCHI	3	R	3′ RACE Adapter	gaccacgcgtatcgatgtcga

instructions. Reverse transcriptions were performed with SUPERSCRIPT III RNase H-Reverse Transcriptase (Invitrogen) with an oligo-(dT) primer. Degenerate primers corresponding to highly conserved regions in insect chitinases were also used to amplify a PCR product corresponding to the *S. frugiperda* chitinase cDNA. The degenerate and the specific primers utilized for *SfCHI* cloning are shown in Table 1. The same procedure used for *SfCHSB* cloning were also employed to assemble the cDNA coding sequence for the entire *SfCHI*, as well as 5′- and 3′-UTR sequences. The 5′-UTR sequence of the *SfCHI* cDNA was determined by utilizing the 5′-RACE system version 2.0 (Invitrogen) according to the manufacturer's instructions. PCR was carried out with an adapter primer and the following gene-specific anti-sense primer: 5′-TGAATGCTCT-CCTGAGCTCC-3′ (positions 507–526 in the cDNA sequence, GenBank accession # AY527414). To reach the 3′-end of cDNA, PCR was carried out by using an oligo-(dT) adapter primer and the gene specific forward primer, 5′-TCGTTCACTCTGTCAGCTGG-3′ (positions 820–839).

To confirm the cDNA sequence assembled from overlapping PCR products, the entire protein coding regions of *SfCHSB* and *SfCHI* were amplified by PCR reactions with the following sets of forward and reverse primers, respectively: 5′-GAATTATCTCGAATGGC GAG-3′ and 5′-GTGGTTATCACGCGAAATGG-3′ for *SfCHSB*, and 5′-CCGCAACACCGCAATTG TTC-3′ and 5′-CACGCACGATTAGTCTAGGG-3′ for *SfCHI*. The PCR cycles were performed as follows: denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and polymerization at 72 °C for 4.5 min using Takara (Japan) *Taq* polymerase for 30 cycles.

The amplified PCR fragments from each reaction were subjected to electrophoresis in 1% agarose gels containing ethidium bromide, and purified using the Freeze 'N Squeeze DNA Gel Extraction Spin Column kit (Bio-Rad). The DNA fragments were subcloned into the TOPO 4 vector (Invitrogen) and sequenced with an automated sequencer (ABI Prism 3700) at the Kansas State University DNA sequencing facility.

2.2. DNA and protein sequence analyses

The sequences of *SfCHSB* and *SfCHI* cDNAs were compared with other sequences deposited in GenBank by using the "BLAST-N" or "BLAST-X" tools at the National Center for Biotechnology Information (NCBI) web site. Alignments of nucleotide sequences and deduced amino acid sequences from cDNA clones were done with the aid of the ClustalW software (PAM250).

2.3. RNA expression analysis by reverse transcription—PCR (RT-PCR)

S. frugiperda larvae were purchased from Benzon Research Lab (Carlisle, PA). Larvae were immobilized by placing them on ice and the guts were dissected in cold 125 mM NaCl. PMs with their contents were then separated from midgut tissue. The epithelia of three-fifth instar larvae or prepupae were divided into three parts of equal length (denoted as anterior, middle and posterior portions of the midgut) and the tissues were

immediately frozen in liquid nitrogen. The tissues were ground using a glass tissue grinder and total RNA was extracted utilizing the RNeasy Mini Kit (Qiagen). Different PCR cycle numbers were previously tested to assure that amplification was carried out in log-phase.

Total RNA was also isolated from the whole midgut and epidermis of third instar larvae, wandering stage larvae, prepupae and pupae. Two micrograms of total RNA were used as templates for first strand cDNA synthesis with an oligo-(dT) primer and the Superscript III RT (Invitrogen). This cDNA was employed as a template for amplification and detection of specific *SfCHSB* and *SfCHI* transcripts. The primers used were: 5′-CGACTGAACACATGATTGGC-3′ (forward) and 5′-TGAACTCTAACGAACCACTC-3′ (reverse) for *SfCHSB*, which generated a 954-bp product, and 5′-CCGCAACACCGCAATTGTTC-3′ (forward) and 5′-CACGCACGATTAGTCTAGGG-3′ (reverse) for *SfCHI*, which generated a 1705-bp product. The primers 3′-ATGAAGCAGGGAGTCCTCAC-5′ (forward) and 3′-GCGTTTGACCTCTTCTTGGC-5′ (reverse) were utilized for monitoring the expression of transcripts corresponding to the control ribosomal protein RpS6, which generated a PCR product of 500 bp. The PCR reaction was carried out in a 20 µl final volume. The PCR program profile (23 or 28 cycles) comprised a denaturing step at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1.5 min. The products of these reactions were visualized after electrophoresis in 1% agarose gel containing ethidium bromide.

2.4. Chitin staining

Midguts from third and fifth instar larvae and wandering and prepupal stages were dissected out and chitin associated with PM was stained with the chitin-binding domain from a chitinase of W12 tagged with fluorescein-isothiocyanate (FITC-CBD, New England BioLabs, Beverly, MA), according to the method of Arakane et al. (2005). Briefly, the midguts from all stages were fixed in 3.7% formaldehyde/PBS (10 mM sodium phosphate buffer, pH 8 containing 100 mM NaCl) for 1 h on ice, followed by washing three times with PBS. For staining, the midguts were incubated for 16 h with the FITC-CBD probe using a 100-fold dilution in PBS pH 8, at room temperature. After washing off the excess probe, midguts from third and fifth instar larvae were cut longitudinally and photographs were taken of PM isolated from the epithelia. Because at wandering and prepupal stages the PM is not intact, instead of PM, whole midguts (with epithelia and after longitudinal dissection) were photographed at these time points. The fluorescence was recorded with a Leica MZ FLIII fluorescence stereomicroscope. Photography was done with a Nikon digital camera, Dxm 1200F. Because no tissue permeation was performed, apparently only the extracellular or cell surface-associated chitin would have been labeled in this protocol.

3. Results

3.1. Cloning and sequencing SfCHSB and SfCHI

Complete cDNA sequences of *SfCHSB* (4.6 kb) and *SfCHI* (1.6 kb) were deposited in the GenBank database with accession numbers of AY525599 and AY527414, respectively. Fig. 1 shows the nucleotide and corresponding amino acid sequences of *SfCHSB* (Fig. 1A) and *SfCHI* (Fig. 1B), where the putative transmembrane segments (predicted by TMHMM program v5. 2.0), potential N- and O-glycosylation sites, and potential signal peptide sequences are indicated.

The deduced amino acid sequence of the catalytic domain of SfCHSB was aligned with the corresponding sequences from other insect chitin synthases. The highest amino acid sequence identity (86%) was to the lepidopteran CHSB from *M. sexta*, whereas a lower level of identity (74%) was seen with CHSA from *M. sexta*. As expected, high amino acid sequence identities were found with other insect class B chitin synthases (Arakane et al., 2004; Hogenkamp et al., 2005) from *T. castaneum* (TcCHS-B, GenBank accession # AAQ55061 (64%), *Anopheles gambiae* (AgCHS-B, GenBank accession # XP_321951 (65%) and *Drosophila melanogaster* (DmCHS-B, GenBank accession # NP_524209) (62%).

Like other insect CHSs, SfCHSB has an N-terminal domain with several membrane-spanning regions, a central domain of high sequence identity/similarity with putative catalytic domains of CHSs of fungi, nematodes and other insects, and a C-terminal domain with additional transmembrane regions characteristic of glycosyltransferase family 2 enzymes (Coutinho and Henrissat, 1999). The mature SfCHSB protein is 1523 amino acids-long with a molecular mass of 174 kDa and a pI of 5.8 (predicted by the sequence analysis tools at www.expasy.ch). The predicted protein contains the catalytically critical sequence containing aspartyl residues as well as the motif QXXRW (Gln–Arg–Arg–Arg–Trp, positions 869–873 in the amino acid sequence), which are conserved in polymerizing β-glycosyltransferases from many species (Saxena et al., 2001).

The predicted amino acid sequence of SfCHI (Fig. 1B) includes an N-terminal catalytic domain and a C-terminal cysteine-rich chitin-binding domain. A serine/threonine-rich linker region connects these two domains, which is probably highly O-glycosylated. The linker region may facilitate secretion from the cell and help stabilize the enzyme in the presence of gut proteolytic enzymes (Arakane et al., 2003). These domains are also present in several chitinases from

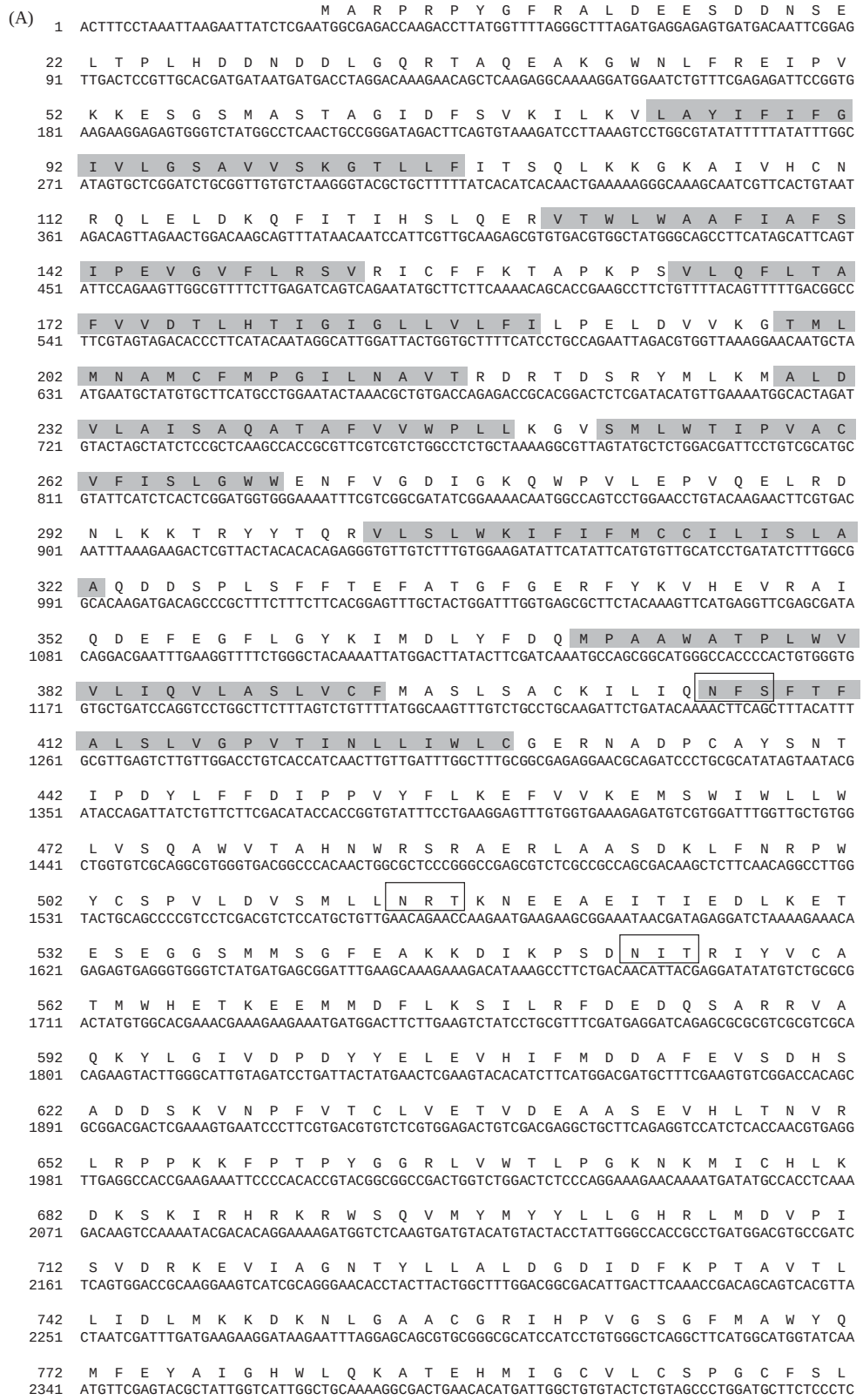

Fig. 1. Nucleotide and deduced amino acid sequences of: SfCHSB (A) and SfCHI (B). The gray boxes indicate putative transmembrane segments. Potential N- and O-glycosylation sites are pointed by open and black boxes, respectively. Potential signal peptide sequence is underlined (panel B).

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 802  F R G K A L M D D N V M K K Y T L T S H E A R H Y V Q Y D Q
2431  TTCAGAGGAAAGGCTTTGATGGACGACAACGTTATGAAGAAATATACCTTAACTTCCCACGAGGCACGACACTATGTGCAATACGATCAA

 832  G E D R W C T L L L Q R G Y R V E Y S A V S D A Y T H C P E
2521  GGCGAGGACCGTTGGTGCACGCTACTGCTGCAGCGCGGGTACCGCGTGGAGTACAGCGCGGTGTCGGACGCGTACACGCACTGCCCCGAG

 862  H F D E F F N Q R R R W V P S T L A N I F D L L G S A K L T
2611  CACTTCGACGAGTTCTTCAACCAGCGCCGCCGCTGGGTGCCCTCCACGCTCGCCAACATCTTCGACCTGCTCGGCAGCGCCAAGCTCACC

 892  V K S N D N I S T L Y I V Y Q F M L I V G T V L G P G T I F
2701  GTCAAGTCCAACGACAACATCTCCACCCTCTATATAGTCTATCAGTTCATGTTGATAGTGGGTACGGTGTTGGGTCCCGGCACGATCTTC

 922  L M M G G A M N A I I Q I S N A Y A M M L N L V P L V I F L
2791  CTGATGATGGGGGGAGCCATGAACGCCATCATTCAGATCAGCAACGCGTACGCGATGATGTTGAACCTCGTACCACTCGTCATCTTCCTT

 952  I V C M T C Q S K T Q L F L A N L I T C A Y A M V M M I V I
2881  ATAGTCTGTATGACTTGTCAGTCAAAGACGCAGCTCTTCCTCGCTAACCTCATAACATGCGCATACGCAATGGTGATGATGATCGTGATA

 982  V G I V L Q I V E D G W L A P S S M F T A L I F G T F F V T
2971  GTGGGGATAGTTCTGCAGATAGTGGAGGATGGATGGCTGGCTCCGTCCAGTATGTTCACAGCTTTAATATTCGGTACATTCTTCGTCACC

1012  A A L H P Q E I K C L L F I A V Y Y V T I P S M Y M L L I I
3061  GCGGCACTACACCCGCAAGAGATCAAATGTTTGTTGTTCATAGCAGTGTACTATGTAACCATCCCTAGTATGTACATGTTGTTGATCATA

1042  Y S I C N L N N V S W G T R E T P Q K K T A K E M E M E Q K
3151  TACTCCATCTGTAATCTCAACAACGTATCCTGGGGTACCAGGGAGACACCGCAGAAGAAAACTGCTAAGGAAATGGAAATGGAACAGAAG

1072  E A E E A K K K M E S Q G L K K L F A K G E E K S G S L E F
3241  GAAGCAGAAGAAGCGAAGAAAAAAATGGAGAGTCAGGGTTTGAAGAAGTTGTTTGCCAAGGGAGAAGAGAAGAGTGGTTCGTTAGAGTTC

1102  S V A G L L R C M C C T N P E D H K D D L N M M Q I S H A L
3331  AGTGTGGCGGGCCTGTTGCGATGTATGTGCTGCACCAATCCAGAGGATCATAAGGACGATCTCAACATGATGCAGATCTCACACGCGTTG

1132  E K I N K R L D Q L D V P P E P T H Q P S H P H T H V E T V
3421  GAGAAGATAAATAAGAGATTGGATCAACTCGATGTCCCTCCTGAGCCGACCCACCAGCCCTCGCATCCGCACACACACGTGGAGACGGTC

1162  G V R D Y E D S E I S T E I P K E E R D D L I N P Y W I E D
3511  GGTGTTCGTGATTACGAAGACAGCGAGATTTCCACTGAAATTCCTAAGGAAGAACGAGACGACCTGATTAACCCCTACTGGATCGAGGAC

1192  V E L Q K G E V D F L T T A E T N F W K D V I D E Y L L P I
3601  GTGGAACTCCAGAAGGGCGAGGTAGACTTCCTCACCACCGCTGAGACCAACTTCTGGAAGGATGTCATCGATGAATACTTACTGCCTATT

1222  D E D K R E I E R I R K D L K N L R D K M V F A F V M L N S
3691  GATGAGGACAAGCGTGAAATTGAACGTATAAGAAAAGATTTGAAGAACTTGCGAGATAAGATGGTGTTTGCGTTCGTGATGTTGAACTCT

1252  L F V L V I F L L Q L S Q D Q L H F K W P F G Q K S S M E Y
3781  CTGTTCGTGCTCGTCATCTTCCTGCTGCAGCTCAGCCAGGACCAGCTGCACTTCAAGTGGCCATTCGGACAGAAGTCCAGCATGGAGTAC

1282  D N D M N M F I I T Q D Y L T L E P I G F V F L L F F G S I
3871  GATAATGATATGAATATGTTCATCATAACCCAAGACTACTTAACGCTGGAACCTATCGGTTTCGTGTTCCTCCTGTTCTTCGGCTCCATC

1312  I M I Q F T A M L F H R L D T L A H L L S T T K L D W Y F S
3961  ATCATGATCCAGTTCACCGCCATGTTGTTCCATCGCCTGGACACGCTGGCCCATCTGCTGTCCACCACCAAGCTGGATTGGTATTTCAGT

1342  K K P D D L S D D A L I D S W A L T I A K D L Q R L N T D D
4051  AAGAAGCCGGACGACCTATCAGACGATGCGCTAATAGACTCTTGGGCGTTGACAATAGCGAAGGATCTTCAACGTCTGAACACCGACGAC

1372  L D K R N N N E H V S R R K T I Y N L E K G K E T K P A V I
4141  TTGGATAAACGAAATAACAACGAACACGTGTCCAGGAGGAAGACCATATATAACTTGGAGAAAGGGAAGGAAACCAAACCGGCTGTTATC

1402  N L D A N A K R R L T I L Q N E D S E L I S R L P S L G P N
4231  AACCTCGATGCCAACGCCAAGAGGAGATTGACTATCCTGCAGAATGAAGACTCAGAATTGATCTCCCGCCTGCCATCTCTGGGACCTAAT

1432  L A T R R A T V R A I N T R R A S V M A E R R R S Q F Q A R
4321  TTGGCAACTCGTCGTGCCACGGTGCGTGCAATAAACACTCGACGCGCATCTGTCATGGCGGAGCGACGCAGGTCTCAGTTCCAAGCGCGA

1462  P S G G S Y M Y N N P Q N T I Q L D D M V G G P S T S G V Y
4411  CCTTCCGGGGGATCATACATGTATAATAACCCTCAAAACACGATTCAGCTGGACGATATGGTCGGGGGGCCGTCGACGTCGGGAGTGTAC

1492  V N R G Y E P A L D S D I E D T P V P T R R S V V H F T D H
4501  GTGAACCGAGGGTACGAGCCCGCCCTGGACAGCGACATCGAGGACACGCCCGTGCCCACCAGACGATCCGTTGTACACTTCACCGACCAT

1522  F A *
4591  TTCGCGTGATAACCACCAAAATCTGACTAACATCTCCATATTACATTTTCTACTCTGTTACGAAACGATAAAGTTTAAAGTGTATTAAAT
4681  AAATTGGACAGATTTGAGTAGGTTTCACGTTTGTGTTTAAATAATTTATGAAAATAACATACCTATTGCTTTATGACCGCTTTAATATTA
4771  AAAGAAACTCAATATATGCATTAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Fig. 1. (*Continued*)

animals and microorganisms (Kramer and Muthukrishnan, 1997, 2005). The N-terminal region consists of a hydrophobic leader peptide (Fig. 1B), followed by a sequence homologous with the catalytic domain of other insect chitinases. Signature sequences that correspond to conserved motifs found in many family 18 chitinases are

(B)

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                                  M  R  A  I  L  A  T  L  P  V  L  A  V  V  T  T  A  I  E  A  D  S
    1 GCCGCAACACGGCAATTGTTCAAAATGAGAGCGATACTGGCGACGTTGCCCGTCCTGGCGGTCGTAACGACTGCAATTGAAGCGGACAGC

   23  K  A  R  I  V  C  Y  F  S  N  W  A  V  Y  R  P  G  V  G  R  Y  G  I  E  D  I  P  V  D  L
   91 AAAGCGCGCATAGTATGCTACTTCAGCAACTGGGCGGTGTATCGACCTGGCGTGGGTCGCTACGGCATCGAGGACATCCCTGTGGACCTC

   53  C  T  H  I  I  Y  S  F  I  G  V  T  E  K  S  N  E  V  L  I  I  D  P  E  L  D  V  D  K  N
  181 TGCACTCATATCATCTACTCTTTTATTGGAGTCACTGAAAAGTCCAATGAAGTTCTTATTATTGACCCTGAGTTGGACGTAGACAAGAAT

   83  G  F  S [N  F  T] A  L  R  K  S  H  P  D  V  K  F  T  V  A  V  G  G  W  A  E  G  G  S  K
  271 GGCTTCAGCAACTTCACAGCTCTTCGCAAGTCGCACCCTGACGTCAAGTTCACCGTGGCTGTCGGTGGCTGGGCTGAAGGAGGATCCAAA

  113  Y  S  H  M  V  A  Q  K  Q  T  R  V  A  F  V  R  S  V  V  D  F  L  K  K  Y  D  F  D  G  L
  361 TACTCCCACATGGTCGCACAGAAACAAACGCGAGTGGCATTTGTTAGGAGCGTTGTTGATTTCTTGAAAAAATACGACTTCGATGGTTTG

  143  D  L  D  W  E  Y  P  G  A  A  D  R  G  G  S  F  S  D  K  D  R  F  L  F  L  V  Q  E  L  R
  451 GACCTGGACTGGGAGTACCCTGGTGCTGCTGACCGTGGTGGTTCCTTCTCCGACAAGGATCGGTTCCTCTTCCTCGTCCAGGAGCTCAGG

  173  R  A  F  I  R  E  K  R  G  W  E  L  T  A  A  V  P  L  A  N  F  R  L  M  E  G  Y  H  V  P
  541 AGAGCATTCATCAGGGAGAAGAGAGGCTGGGAACTGACTGCTGCTGTGCCACTCGCCAACTTTAGGCTGATGGAAGGTTACCACGTACCT

  203  D  L  C  Q  E  L  D  A  I  H  V  M  S  Y  D  L  R  G  N  W  A  G  F  A  D  V  H  S  P  L
  631 GATCTTTGCCAGGAGCTGGATGCTATACATGTGATGTCGTACGACCTGAGAGGAAACTGGGCTGGATTCGCTGATGTGCACTCGCCGTTG

  233  Y  K  R  P  H  D  Q  W  A  Y  E  K  L  N  V  N  D  G  L  A  L  W  E  E  K  G  C  P  S  N
  721 TACAAGCGTCCCCATGACCAGTGGGCTTATGAGAAACTGAATGTTAACGATGGTTTAGCTCTCTGGGAAGAAAAGGGCTGTCCCAGTAAC

  263  K  L  V  V  G  I  P  F  Y  G  R  S  F  T  L  S  A  G  N  N  N  Y  G  L  G  T  Y  I  N  K
  811 AAGTTGGTGGTCGGTATCCCGTTCTACGGCCGTTCGTTCACTCTGTCAGCTGGTAACAACAACTACGGCCTCGGCACTTACATCAACAAG

  293  E  A  G  G  G  D  P  A  P  Y  T [N  A  T] G  F  W  A  Y  Y  E  I  C  T  E  V  D  K  E  G
  901 GAGGCTGGAGGTGGTGACCCAGCTCCTTACACCAACGCTACTGGATTCTGGGCTTACTACGAGATCTGTACCGAAGTCGACAAAGAAGGT

  323  S  G  W  T  K  K  W  D  E  H  G  K  C  P  Y  A  Y  K  G  T  Q  W  V  G  Y  E  D  P  R  G
  991 TCAGGATGGACTAAGAAGTGGGACGAGCATGGCAAGTGCCCCTACGCCTACAAGGGAACCCAGTGGGTCGGTTACGAAGACCCCCGCGGT

  353  V  E  I  K  M  N  W  I  K  E  K  G  Y  L  G  A  M  T  W  A  I  D  M  D  D  F  K  G  L  C
 1081 GTGGAGATCAAGATGAACTGGATCAAGGAGAAGGGATACCTCGGTGCTATGACCTGGGCTATTGACATGGATGACTTCAAAGGTCTTTGC

  383  G  D  E  N  P  L  I  K  L  L  H  K  H  M  S  T  Y  T  V  P  P  P  R [S] G  N [T][T] P [T]
 1171 GGTGATGAGAATCCTCTTATCAAGCTCCTCCACAAACATATGAGCACCTACACCGTCCCACCACCACGCTCTGGAAATACCACTCCTACG

  413  P  E  W  A  R  P  P [S][T][T][S] G  P [S] E  G  E  P  I  V [T][T] A  R  P [T][T][T][T] K
 1261 CCTGAATGGGCGCGCCCGCCGTCGACGACGTCCGGCCCGTCGGAGGGAGAGCCAATCGTGACGACTGCAAGGCCGACCACGACCACTAAG

  443  R  P  M  K  Q [T][T] T  S  K  P  Q  V  V  I  E  D  D  E  F  D  I  A  V  R  P  E  P  P  K
 1351 CGGCCTATGAAGCAGACGACCACTTCAAAGCCTCAAGTCGTTATTGAAGATGATGAATTTGATATTGCCGTGAGACCTGAACCACCTAAG

  473  A  P  E [T] P  V  V  P  E [S] P  E  A  P  E  S  P  A  E  N  E  I  D  D  H  D  V  C  N  S
 1441 GCACCTGAGACACCAGTGGTCCCCGAATCCCCTGAAGCCCCAGAATCCCCTGCTGAGAATGAGATCGACGACCATGATGTCTGCAACTCT

  503  E  E  D  Y  V  P  D  K  K  K  C  T  K  Y  W  R  C  V  N  G  K  G  M  Q  F  T  C  H  P  G
 1531 GAAGAAGATTATGTCCCAGATAAGAAGAAATGCACCAAGTACTGGCGCTGCGTCAACGGAAAGGGAATGCAGTTCACCTGCCACCCAGGA

  533  T  M  F  N  T  Q  L  N  V  C  D  W  P  D  N  A  K  R  Q  D  C  E  P  E  *
 1621 ACAATGTTCAACACGCAACTGAACGTTTGCGATTGGCCCGACAACGCTAAACGTCAAGACTGCGAGCCCGAATAGGGCGCTCTACTGGTC
 1711 ATTGCAGAGATACCGTTTGATCGAAATAGCATTCTGCTTGCAAGACAGCTGATAAATTGCGTGGTAGTGCTGTGGGTTGCAAGCCGCTAT
 1801 CACTGGTGACTAGACATGCATGTAGAGACATAGCTGTCACTAAAGAAGCAATTAGCAAATGAGCATCTGTCACCCCACATGAAGCCACGC
 1891 TGCAGATTATTCTCTAATGTCTATAAATCCAACATCTGTTTTGCAAGACTCAATTTTGTTAGCTTTATAAAGCTACCATTATTTCCCCTA
 1981 TTAACTCGGCGTTATAAAAATATTAGTCATCTCTATGGGAACACAAAGGGCCGCTAATAAACCTTTGTGTGGTCAGCCGTTGAGTGGATT
 2071 TTGATAAAATGGTATCTTTCCTTTACGCAATGAATCATAATAATAGACGTATTTACGAAGTTTTAGGTTACTGAACAATATTGTCGTATT
 2161 TTGTTATGCAGTAACATAATACGCCAATGTTTTCTTGTCTATAGTTTGTAACAGGATAGGATTATTTGTGATGATAGGCTAGACATTTAC
 2251 GGTTGTAAAAAAAAAAAAAAAAAA
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Fig. 1. (*Continued*)

shown in Fig. 1B and include KFMVAVGGWAEGS and YDFDGLDLWEYP stretches found near or in the catalytic site (Watanabe et al., 1993; Kramer and Muthukrishnan, 2005). In addition to these two regions with the highest sequence similarity, the C-terminal region of SfCHI contains a conserved chitin-binding

domain (carbohydrate-binding module family 14, Coutinho and Henrissat, 1999; Tellam, 1996; Tjoelker et al., 2000), which includes six conserved cysteines. This motif is not only found in other insect and nematode chitinases, but also in some plant chitinases and related proteins (Verheyden et al., 1995), as well as in several PM proteins (Tellam, 1996). The amino acid sequence similarity among the peritrophin chitin-binding domains is not very high, but the positions of the cysteines are highly conserved ($CX_{8-9}CX_{17-21}CX_{10-11}CX_{12-13}CX_{11}C$, where X may be any amino acid residue except cysteine) (Tellam et al., 1999). The SfCHI chitin-binding domain sequence is $CX_{12}CX_5CX_9CX_{12}CX_{10}C$, which is similar to the peritrophin chitin-binding domain. The length of the mature SfCHI protein is 555 amino acids, corresponding to a molecular mass of approximately 62 kDa and a pI of 5.3, which are similar to values determined for other insect chitinases (Kramer and Muthukrishnan, 1997). The encoded amino acid sequence of SfCHI was aligned with three other lepidopteran chitinase sequences from *S. litura* (Shinoda et al., 2001), *M. sexta* (Kramer et al., 1993) and the tomato moth, *Lacanobia oleracea* (Fitches et al., 2004), together with a midgut chitinase from the sand fly, *Lutzomyia longipalpis* (Ramalho-Ortigao and Traub-Cseko, 2003). The alignment indicates that the highest sequence identity is in the central domain (positions 21–396 in the amino acid sequence), which includes the region responsible for catalytic activity. SfCHI exhibits 91% sequence identity with *S. litura* chitinase, 85% with *L. oleracea* chitinase, and 84% with *M. sexta* chitinase but only 36% with *L. longipalpis* chitinase.

3.2. Expression of SfCHSB and SfCHI transcripts during development of S. frugiperda

RT-PCR experiments were carried out to analyze the expression patterns of *SfCHSB* and *SfCHI* in the midgut during development of *S. frugiperda*. Fig. 2 shows that *SfCHSB* is highly expressed during feeding (third and fifth larval instars). Trace amounts of *SfCHSB* transcripts were detected in the wandering and prepupal stages. No PCR product with the size expected for *SfCHSB* transcript was observed with cDNA from the epidermis at any developmental stage (Fig. 2). *SfCHI* transcripts were detected in both midgut and epidermis in wandering larvae, prepupae and pupae, and the peak of *SfCHI* expression in the epidermis occurred at the prepupal stage. There was no trace of chitinase transcripts in these tissues during the feeding stages (third and fifth instars). The control experiment showed that the levels of transcripts corresponding to the ribosomal protein, RpS6, were approximately the same in all samples of midgut and epidermis at all of the developmental stages examined. Although the PCR data are only semi-quantitative, these results indicate that there are qualitative differences in the developmental patterns of expression of *SfCHSB* and *SfCHI* genes in the midgut. *SfCHSB* and *SfCHI* transcripts are detected in anterior, medium and posterior portions of the midgut (data not shown).

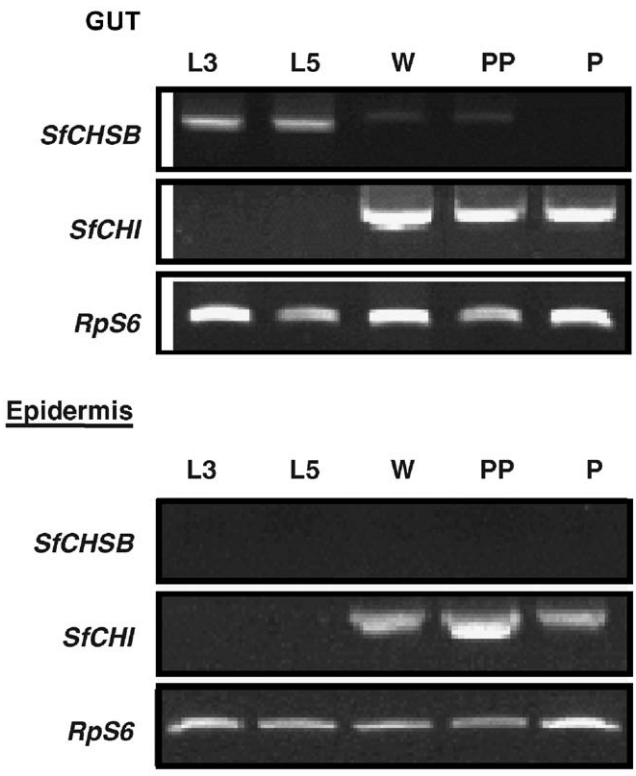

Fig. 2. RT-PCR analysis of the expression of *SfCHSB* and *SfCHI* in the midgut and epidermis of *S. frugiperda* at various stages of development including third (3) and fifth (5) instar larvae, wandering stage (W), prepupae (PP) and pupae (P). Primers for the constitutively expressed housekeeping gene, *RpS6*, were used as control. cDNAs prepared from approximately 2 μg of RNA were used as templates for PCR reactions using gene-specific primers as described in Section 2. After several trials to assure log-phase amplification, 23, 28 and 23 PCR cycles were carried out to amplify the fragments corresponding to *SfCHSB*, *SfCHI* and *RpS6* genes, respectively.

3.3. Presence of chitin in the PM during development

A specific chitin-binding domain fragment of a chitinase from *Bacillus circulans* WL-12 coupled with FITC was used to detect chitin in the PM of *S. frugiperda* during development. Feeding stage larval PMs had high FITC fluorescence due to chitin, whereas the guts from wandering stage larvae and prepupae had no detectable fluorescence indicating the absence of chitin-containing structures such as the PM at these developmental stages (Fig. 3). This observation was confirmed by visualization of the midgut contents by dissection. There were no PMs in larvae exhibiting wandering behavior or in prepupae.

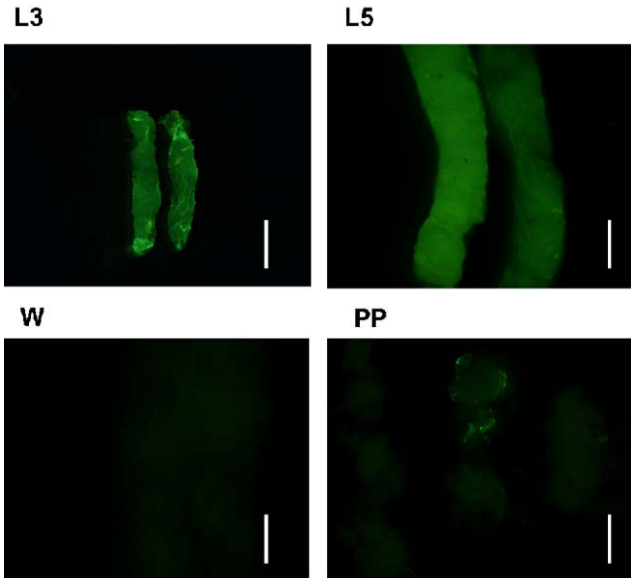

Fig. 3. Chitin staining of the putative peritrophic membrane using FITC-CBD probe. Midguts of insects at the indicated developmental stage were stained with FITC-CBD as described in Section 2. Chitin was visualized in PMs of third (L3) and fifth (L5) instar larval stages but not in wandering (W) and pre-pupal (PP) stages, when chitinase is expressed. Fluorescence was observed using a fluorescence microscope equipped with appropriate filter (see Section 2). Bars = 0.5 cm.

4. Discussion

The *SfCHSB* gene reported in this paper is from the midgut and shares greater sequence similarity with other insect *CHSBs* than with *CHSAs*, suggesting that this gene belongs to the *CHSB* family. Similarly, the amino acid sequence of the encoded protein shows greater similarity to other insect class B CHSs than to class A CHSs. The RT-PCR data presented in this paper provide experimental support for the hypothesis that the synthesis of chitin for the PM may be a major function of class B CHSs, since the *SfCHB* transcripts are present in the midgut during feeding stages, but absent in epidermis (Fig. 2).

SfCHI cDNA encodes the domains found in several other chitinases including the catalytic domain, cysteine-rich chitin-binding domain and a serine–threonine-rich linker region (Tellam, 1996; Arakane et al., 2003). The structure of the encoded protein predicted by homology modeling is very similar to those of other chitinases from insects and nematodes (Kramer and Muthukrishnan, 2005). Some chitinases from plants and other proteins with no chitinase activity (lectins from plants and peritrophins from insects) also have chitin-binding domains containing several cysteines, but these domains may be located near either the N- or C-terminal ends of these proteins (Blaxter, 1996). The function of these domains in chitinases is presumably to anchor the enzyme tightly onto the large insoluble polymeric substrate, thereby facilitating the heterogeneous hydrolytic process (Arakane et al., 2003). In peritrophins, the putative function of these domains is also to anchor the protein to chitin, maintaining the PM structure (Tellam, 1996).

Chitin is present in the PM lining the midguts of *S. frugiperda* feeding larvae but is absent in wandering and prepupal stages. Terra (2001) proposed a simple methodology to verify the presence of a PM, which is based on the ability to grab the gut content-lining structure with a pair of forceps. In the case of larvae in the feeding stages, the PM was easily manipulated with a pair of forceps. However, when the larvae were in the wandering or prepupal stage, no midgut content lining could be picked up with the forceps. These observations were consistent with the results obtained using a FITC-conjugated chitin-binding domain reporter protein described in the present study. Chitin was visualized in the gut of feeding stage larvae, in the PM, whereas there was little or no chitin labeling in the midguts of wandering and prepupal stage insects, suggesting the absence of a PM.

The inverse patterns of expression of the *SfCHSB* and *SfCHI* genes in the insect gut during development are consistent with opposing physiological functions for these enzymes. The high levels of *SfCHSB* mRNA in fifth instar larvae and its absence during the pupal stage may indicate a role for the SfCHSB protein in the production of the chitin-rich PM. During feeding stages, the insect is actively digesting food and needs this structure to protect the cells of the gut lining and to increase the efficiency of nutrient digestion (Terra, 2001; Terra and Ferreira, 2005). The transcripts for *SfCHI* were not detected during the feeding stage, but they were found in wandering, pre-pupal and pupal stages in both gut and epidermal tissues (Fig. 2). The tight developmental regulation of chitinase expression suggests that the presence/absence of this enzyme might be detrimental to insect growth if not expressed at appropriate times. Also, plants or microorganisms expressing chitinases might be resistant to insects, because exposure to this enzyme during feeding may digest their PM (Kramer et al., 1997; Otsu et al., 2003; Fitches et al., 2004; Lertcanawanichakul et al., 2004; Thamthiankul et al., 2004), decreasing the efficiency of digestion and allowing the entry of pathogens into insect tissues (Brandt et al., 1978; Pechan et al., 2002; Rao et al., 2004).

Therefore, we propose here that the expression of *SfCHSB* and *SfCHI* genes is precisely coordinated to control the synthesis of the PM during the feeding stage and its degradation during larval–larval and larval–pupal molts and this is accomplished at the level of mRNA transcription. Whether the regulation of *CHS* and *CHI* expression in the armyworm midgut is coupled or independently regulated, as is the case for some yeast

species (Selvaggini et al., 2004), is not known and will be the focus of future studies.

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